

Julie Gale

PC 1/002/001754

Characterization of Copper Interactions with Alzheimer Amyloid β Peptides: Identification of an Attomolar-Affinity Copper Binding Site on Amyloid β 1–42

*†‡Craig S. Atwood, *†‡Richard C. Scarpa, *†‡Xudong Huang, ‡§Robert D. Moir,
*†‡Walton D. Jones, ||David P. Fairlie, ‡§Rudolph E. Tanzi, and *†‡Ashley I. Bush

*Laboratory for Oxidation Biology, Departments of †Psychiatry and §Neurology, and ‡Genetics and Aging Unit, Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, U.S.A.; and ||Centre for Drug Design and Development, University of Queensland, Brisbane, Queensland, Australia

Abstract: Cu and Zn have been shown to accumulate in the brains of Alzheimer's disease patients. We have previously reported that Cu^{2+} and Zn^{2+} bind amyloid β ($\text{A}\beta$), explaining their enrichment in plaque pathology. Here we detail the stoichiometries and binding affinities of multiple cooperative Cu^{2+} -binding sites on synthetic $\text{A}\beta$ 1–40 and $\text{A}\beta$ 1–42. We have developed a ligand displacement technique (competitive metal capture analysis) that uses metal-chelator complexes to evaluate metal ion binding to $\text{A}\beta$, a notoriously self-aggregating peptide. This analysis indicated that there is a very-high-affinity Cu^{2+} -binding site on $\text{A}\beta$ 1–42 ($\log K_{\text{app}} = 17.2$) that mediates peptide precipitation and that the tendency of this peptide to self-aggregate in aqueous solutions is due to the presence of trace Cu^{2+} contamination (customarily $\sim 0.1 \mu\text{M}$). In contrast, $\text{A}\beta$ 1–40 has much lower affinity for Cu^{2+} at this site (estimated $\log K_{\text{app}} = 10.3$), explaining why this peptide is less self-aggregating. The greater Cu^{2+} -binding affinity of $\text{A}\beta$ 1–42 compared with $\text{A}\beta$ 1–40 is associated with significantly diminished negative cooperativity. The role of trace metal contamination in inducing $\text{A}\beta$ precipitation was confirmed by the demonstration that $\text{A}\beta$ peptide ($10 \mu\text{M}$) remained soluble for 5 days only in the presence of high-affinity Cu^{2+} -selective chelators. **Key Words:** Human amyloid β peptide—Copper—Affinity—Stoichiometry—Alzheimer's disease—Binding affinity method—Chelators.

J. Neurochem. 75, 1219–1233 (2000).

Alzheimer's disease (AD) is characterized pathologically by the deposition of amyloid plaques and neurofibrillary tangles and by neuronal degeneration in the brains of affected individuals. Amyloid deposits are composed primarily of the amyloid β ($\text{A}\beta$) protein (Masters et al., 1985; Roher et al., 1996) generated as a mixture of polypeptides manifesting carboxyl- and amino-terminal heterogeneity. The $\text{A}\beta$ 1–40 isoform is the predominant soluble species in biological fluids (Shoji et al., 1992; Vigo-Pelfrey et al., 1993). Although less

abundant in biological fluids, $\text{A}\beta$ 1–42 is the predominant species found in plaque deposits (Masters et al., 1985; Roher et al., 1996).

Metal ion homeostasis is severely dysregulated in AD (Hershey et al., 1983; Ehmann et al., 1986; Thompson et al., 1988; Vance et al., 1990; Basun et al., 1991; Samudralwar et al., 1995; Deibel et al., 1996; Cornett et al., 1998; Lovell et al., 1998; González et al., 1999). Although the transition metal ions Cu, Fe, and Zn are maintained at high concentrations within the healthy brain neocortical parenchyma (total dry weight concentrations of 70, 340, and $350 \mu\text{M}$, respectively), increased concentrations of these metal ions are detected in the neuropil of the AD-affected brain, where they are highly concentrated within amyloid plaque deposits with total concentrations reaching ~ 0.4 and $\sim 1 \text{ mM}$ for Cu and Fe/Zn, respectively (Lovell et al., 1998). An elevated Zn^{2+} concentration also can be detected in plaque deposits histologically (Suh et al., 2000). We previously found that $\text{A}\beta$ avidly binds Cu^{2+} , Zn^{2+} , and Fe^{3+} (Bush et al., 1994a,b, 1995; Huang et al., 1997; Atwood et al., 1998), perhaps explaining the recruitment of these metals into amyloid plaque pathology.

Evidence for an interaction between Cu^{2+} and $\text{A}\beta$ 1–40 was first observed by the stabilization of an

Resubmitted manuscript received April 4, 2000; accepted April 18, 2000.

Address correspondence and reprint requests to Dr. A. I. Bush at Laboratory for Oxidation Biology, Genetics and Aging Unit, Neuroscience Center, Massachusetts General Hospital East, Building 149, 13th Street, Charlestown, MA 02129-9142, U.S.A. E-mail: bush@helix.mgh.harvard.edu

Drs. C. S. Atwood and R. C. Scarpa contributed equally to this article.

Abbreviations used: $\text{A}\beta$, amyloid β ; AD, Alzheimer's disease; CDTA, *trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid; CMCA, competitive metal capture analysis; DSA, dog serum albumin; DTPA, diethylenetriaminepentaacetic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate.

TABLE 1. Log K_{app} of Cu $^{2+}$ - and Zn $^{2+}$ -selective chelators

Chelator	Cu $^{2+}$ log K_{app}			Chelator	Zn $^{2+}$ log K_{app}		
	pH 7.4	pH 7.0	pH 6.6		pH 7.4	pH 7.0	pH 6.6
Tris	1.8	1.4	1.0	Tris	3.2	2.8	2.4
Glycine	5.9	5.5	5.1	Bicine	4.7	4.3	3.9
Arginine	5.9	5.5	5.1	Histidine	5.0	4.5	4.2
Methionine	6.3	5.9	5.5	ACES	5.0	4.6	4.2
Asparagine	6.6	6.2	5.8	Citric acid	5.3	4.9	4.5
Histamine	7.2	6.8	6.4	Bipyridyl	5.8	5.4	5.0
Bicine	7.4	7.0	6.6	Tricine	6.5	6.1	5.7
Ethylenediamine	7.8	7.4	7.0	HIMDA	7.1	6.7	6.3
Histidine	8.4	8.0	7.8	NTA	8.0	7.6	7.2
Bipyridyl	8.9	8.5	8.1	EGTA	10.0	9.6	9.2
HIMDA	10.6	10.2	9.8				
EDDA	14.1	13.7	13.3				
EDTA	15.9	15.5	15.1				
DTPA	16.3	15.9	15.5				
CDTA	17.0	16.6	16.2				

ACES, *N*-(carbamoylmethyl)-2-aminoethanesulfonic acid; bicine, *N,N*-bis(2-hydroxyethyl)glycine; bipyridyl, 2,2'-dipyridyl; EDDA, ethylenediamine-*N,N'*-diacetic acid; HIMDA, *N*-(2-hydroxyethyl)iminodiacetic acid; NTA, nitrilotriacetic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

Determination of apparent log K from absolute log K

Metal ion chelators were chosen from the National Institute of Standards and Technology Standard Reference Database 46 (Critically Selected Stability Constants of Metal Complexes Database, version 4.0; U.S. Department of Commerce) and other reference databases (Dawson et al., 1986). Chelators were chosen to give a range of stability constants at approximately half log order intervals. Stability constants ($K = [ML]/[M][L]$, where M = metal ion and L = ligand) chosen for metal:chelators were for 25°C, an ionic strength of 0.1–0.2 M, and a molar ratio of chelator to metal ion of 1:1. Where more than one complex is formed between a metal ion and a chelator, a chelator was only elected if the log K of its metal:chelator complex with a ratio of 1:1 was the greatest of all possible complexes.

The log K of a chelator for metal ions is affected by the pH of the solution as determined by the following equations [after Ringblom (1963) and Schwarzenbach and Flaschka (1969)]:

$$\log K_{apparent} = \log K - \log \alpha$$

where

$$\alpha = \frac{[H^+]_0}{K_{a1} * K_{a2} * K_{a3} * \dots * K_{an}} + \frac{[H^+]_0^{-1}}{K_{a1} * K_{a2} * \dots * K_{an-1}} + \dots + \frac{[H^+]_0}{K_{a1}} + 1$$

$K_a = 10^x$, where $x = -pK_a$ of the chelator (K_a values are listed in order of decreasing pK_a values), and n = no. of pK_a values. Values for log K_{app} for chelators used at pH 7.4, 7.0, and 6.6 are presented in Table 1.

Stoichiometry and binding analyses of metal ions for A β and DSA

Solutions of metal:chelator were prepared in a ratio of 1:2 (5 mM:10 mM) in doubly deionized water and allowed to come to equilibrium for 24 h at room temperature. Samples containing A β (10 μ M) or DSA (10 μ M) with and without metal ion:

chelator complexes (50 μ M metal ion, 100 μ M chelator), along with background controls of protein plus chelator (no metal) or metal:chelator alone, were prepared in 20 mM Tris/150 mM NaCl buffer at pH 7.4 and 6.6 (for A β) and pH 7.0 (for DSA) to a final volume of 1 ml and incubated for 24 h at 37°C. Tris was chosen as the buffer because it only weakly complexes Cu ions and its log K_{app} has been characterized (Dawson et al., 1986). The value of pH 6.6 was chosen as a condition of severe acidosis and also to make comparisons with our previous publication.

Following incubation the sample was split into three fractions: Fraction 1 was used to confirm the initial concentrations of total protein [150 μ l for Micro BCA assay (Pierce, Rockford, IL, U.S.A.)] and total metal ion (150 and 20 μ l for Cu and Zn assays, respectively) in the reaction samples. Fraction 2 (330 μ l) was loaded into a 3-kDa cutoff filter (Concentrator; Amicon, Beverly, MA, U.S.A.) and centrifuged at 10,000 g for 30–45 min, and the filtrate, which contained no peptide, was collected for metal ion analysis (20 μ l for Zn and 160 μ l for Cu). Fraction 3, the remainder of the reaction sample (330 μ l), was centrifuged for 10 min at 10,000 g , and the supernatant was assayed for soluble protein and metal ion concentrations from which the respective concentrations in the pellet could be deduced. We have previously shown that centrifugation of A β solutions at 10,000 g for 10 min sediments all precipitated A β compared with ultracentrifugation at 100,000 g for 1 h (Atwood et al., 1998). As DSA did not precipitate in the presence of Zn $^{2+}$ or Cu $^{2+}$, reaction mixtures of DSA and metal ions were divided into fractions 1 and 2 for assay. Appropriate blank samples (buffer with or without A β , buffer with or without metal ion) also were analyzed for background readings.

Scatchard analysis

Having assayed the amount of protein-bound metal ion in the incubation, the remaining metal ion in the non-protein-bound fraction is in an equilibrium between being free and being bound to the chelator. Therefore, the free concentration of metal ion could be calculated using the chelator stability constant for the metal ion applied to this fraction. Scatchard linearization analysis was only performed on data where the

gate by incubation in 20 mM Tris/150 mM NaCl buffer at pH 7.4 or 6.6 with CuCl₂ (20 μ M) for 1 h at 37°C. Following precipitation, samples were incubated with no chelator, EDTA, or DTPA (200 μ M each) for 1 h at 37°C. Samples were then centrifuged (10,000 *g* for 20 min), and the supernatant was analyzed for protein concentration to determine percent total peptide in the soluble fraction as previously described (Atwood et al., 1998).

Competition analyses of zinc and copper for A β

A β 1–40 (10 μ M) was incubated in 20 mM Tris/150 mM NaCl buffer at pH 7.4 or 6.6 with ZnCl₂, Cu(NO₃)₂, or ZnCl₂ plus Cu(NO₃)₂ (50 μ M each) for 48 h or ZnCl₂ (50 μ M) for 24 h, and then Cu(NO₃)₂ (50 μ M) was added for a further 24 h. Following incubation, samples were analyzed for percent precipitation and stoichiometry of metal ion binding to A β as described above.

SDS-resistant polymerization of A β

A β stock solutions were diluted to 2.5 μ M in phosphate-buffered saline (66 mM phosphate and 150 mM NaCl, pH 7.4) and incubated with or without CuCl₂ (30 μ M) for 0 and 1 day at 37°C. Aliquots of each reaction mixture (2 ng of peptide) were collected into 15 μ l of sample buffer (containing 4% SDS and 5% β -mercaptoethanol) and heated to 95°C for 5 min. Samples were loaded, electrophoresed on polyacrylamide gel electrophoresis (PAGE; Tricine gels, 10–20%; Novex, San Diego, CA, U.S.A.) at 25°C and pH 8.45, transferred to polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, U.S.A.), fixed with glutaraldehyde (1%, vol/vol), blocked with milk (10%, wt/vol), and then probed with anti-A β monoclonal antibody 6E10 (or 4G8; Senetek, Maryland Heights, MI, U.S.A.) overnight at 4°C. The blot was then incubated with anti-mouse horseradish peroxidase conjugate (Pierce) for 2 h at room temperature and developed with ECL reagent (1 min; Amersham, Little Chalfont, Bucks, U.K.) or Supersignal Ultra (5 min; Pierce) following the manufacturer's instructions. The chemiluminescent signal was captured on film or for 10 min at maximal sensitivity using the Fluoro-S Image Analysis System (Bio-Rad). Electronic images were analyzed using Multi-Analyst Software (Bio-Rad). Molecular size markers were from Amersham (Arlington Heights, IL, U.S.A.).

RESULTS

Stoichiometry and estimated binding affinities of Cu²⁺ and Zn²⁺ for DSA

We developed a method (CMCA) to enable the determination of stoichiometry of metal ion binding to both soluble and precipitated forms of A β and to permit an estimation of metal ion binding affinities to A β (Fig. 1). To validate this methodology, we first studied a protein with known Cu²⁺ and Zn²⁺ binding affinities (DSA) (Fig. 2). DSA was found to bind on average \sim 2 Zn²⁺ (1.7 \pm 0.2; mean \pm SD) in the presence of Zn²⁺:chelator complexes with log *K*_{app} values of \leq 6.2 at pH 7.0 (Fig. 2A). Scatchard analysis of Zn²⁺ binding to DSA indicated that there was a single affinity binding site (*K*_D = 1.3 \times 10^{−7} M, log *K*_{app} = 6.9) binding both Zn²⁺ ions (Fig. 2B), in agreement with previously reported values (Masuoka et al., 1993).

DSA also bound \sim 2 Cu²⁺ ions (1.7 \pm 0.1; mean \pm SD) when incubated in the presence of Cu²⁺:chelator

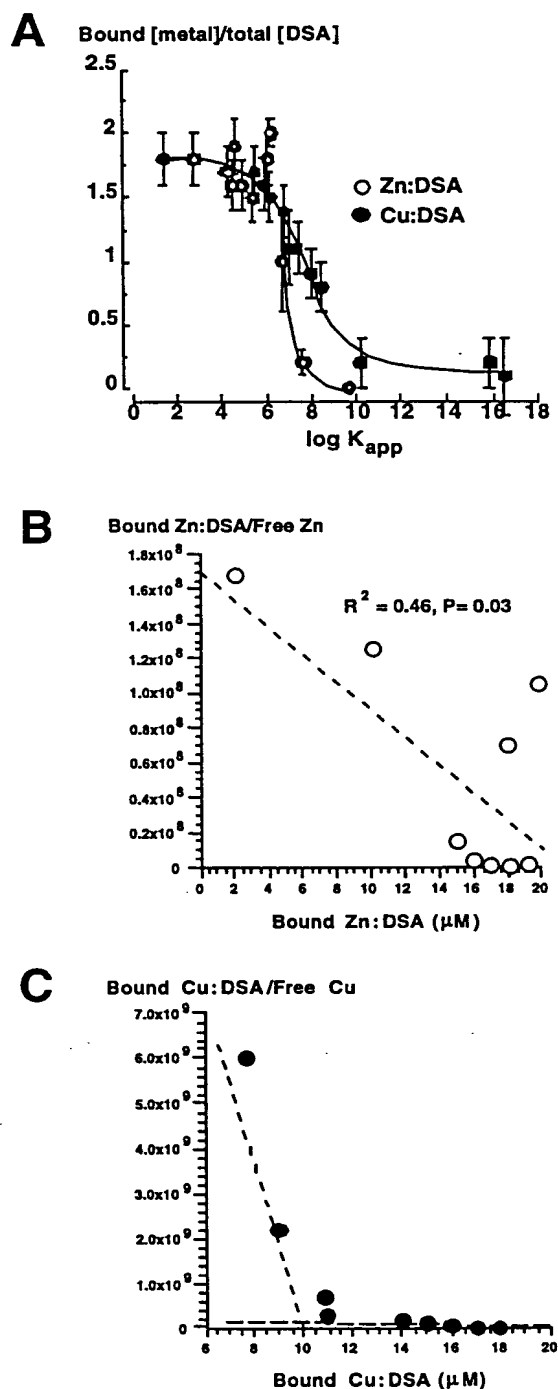


FIG. 2. CMCA of Cu and Zn binding to DSA. **A**: DSA (10 μ M) was incubated with metal:chelator complexes (50 μ M) for 24 h, and the stoichiometry of binding was determined. Data are mean \pm SD (bars) values (*n* = 6). **B** and **C**: Scatchard analysis of the data in **A**.

TABLE 2. Summary of binding affinities ($\log K_{app}$) for Cu²⁺ binding to A β 1-40 and A β 1-42

	A β 1-40		A β 1-42		<i>t</i>	<i>df</i>	<i>p</i>
	$\log K_{app}$	Stoichiometry of Cu:A β	$\log K_{app}$	Stoichiometry of Cu:A β			
pH 7.4							
Highest affinity	10.3	0.5	17.2	0.5			
Lowest affinity	7.9	2	8.3	2			
	Slope $[\log(B/F)]/B$	R^2	Slope $[\log(B/F)]/B$	R^2			
	-0.20	0.93	-0.50	0.88	2.98	44	0.002
pH 6.6							
Highest affinity	9.6	0.5	16.3	0.5			
Lowest affinity	7.0	2.5	7.5	2.5			
	Slope $[\log(B/F)]/B$	R^2	Slope $[\log(B/F)]/B$	R^2			
	-0.15	0.92	-0.42	0.90	3.03	44	0.002

The data in Fig. 3 for Cu²⁺ binding to A β were analyzed and are summarized here. $\log K_{app}$ values are derived from the slopes of the Scatchard plots in Fig. 3E, and the estimated stoichiometries of Cu²⁺ binding to the highest- and lowest-affinity sites are derived from the estimated x-axis intercepts in Fig. 3E. The slope of the analysis of $[\log(B/F)]/B$ derived from the insets of Fig. 3E is shown, along with the regression coefficients of these slopes, reflecting cooperative binding of Cu²⁺ to A β . The slope of $[\log(B/F)]/B$ for Cu²⁺ binding to A β 1-42 is significantly greater than the slope of $[\log(B/F)]/B$ for Cu²⁺ binding to A β 1-40 [*t* test value, degrees of freedom (*df*), and *p* values are shown]. This indicates that there is significantly greater cooperative binding of Cu²⁺ to A β 1-42 compared with A β 1-40.

plexes except in the presence of the chelator with the highest affinity for Cu²⁺ [*trans*-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA), $\log K_{app} = 17.0$], when the solubility of the peptide abruptly and dramatically increased (Fig. 3B). The possibility of CDTA directly binding to A β 1-42 and acting as a chain breaker was excluded by nuclear magnetic resonance spectroscopy studies (X. Huang, K. Barnham, C. S. Atwood, R. E. Tanzi, and A. I. Bush, unpublished data).

At pH 6.6, the stoichiometry of low-affinity Cu²⁺ binding to both peptides increased (Fig. 3C). At pH 6.6, A β 1-40 and A β 1-42 bound 0.8 and 0.3 more Cu²⁺ ions (2.6 ± 0.1 and 2.5 ± 0.1 , respectively) than at pH 7.4 in the presence of Cu²⁺:chelator complexes in lower-affinity range ($\log K_{app} < 7.8$), so that the stoichiometry of Cu²⁺ binding to A β 1-40 approached that of A β 1-42 (Fig. 3C). At pH 6.6, the affinity of the lowest-affinity Cu²⁺ binding site estimated by Scatchard analysis (Fig. 3E) decreased for both A β 1-40 and A β 1-42 (A β 1-40, $K_D = 9.3 \times 10^{-8}$ M, $\log K_{app} = 7.0$; A β 1-42, $K_D = 3.3 \times 10^{-8}$ M, $\log K_{app} = 7.5$; Table 2), compared with the affinity at pH 7.4. There was no significant change in Cu²⁺:A β stoichiometry after incubation with Cu²⁺:chelator complexes in the high-affinity range ($\log K_{app} = 7.8$ –16.2) at pH 6.6 compared with the values at pH 7.4. There was an accompanying decrease of ≈ 1 log unit in the estimated $\log K_{app}$ values for the high-affinity Cu²⁺ binding sites for both A β 1-40 ($K_D = 2.6 \times 10^{-10}$ M, $\log K_{app} = 9.6$; Table 2) and A β 1-42 ($K_D = 4.5 \times 10^{-17}$ M, $\log K_{app} = 16.3$; Table 2) at pH 6.6 (Fig. 3C and E), compared with the corresponding values at pH 7.4 (Fig. 3A and E).

The slope of the plot of $\log(\text{bound [Cu}^{2+}]/\text{free [Cu}^{2+}])$ and bound [Cu²⁺] (Fig. 3E, insets) correlates with the cooperativity coefficient for the multiple Cu²⁺ binding sites on A β . We found that at either pH 6.6 or 7.4, this slope was significantly (≈ 2.5 -fold, *p* = 0.002) greater for Cu²⁺ binding to A β 1-42 than for binding to

A β 1-40 (Fig. 3E and Table 2). This means that the acceleration of the Scatchard plot was 2.5-fold greater for Cu²⁺ binding to A β 1-42, indicating that A β 1-42 has markedly greater cooperative binding of Cu²⁺ than A β 1-40.

The increase in low-affinity Cu²⁺ binding to A β 1-40 induced by incubation at the lower pH (6.6) correlated with a $\approx 30\%$ increase in A β 1-40 precipitation (Fig. 3D) compared with that induced at pH 7.4 (Fig. 3B). However, although the incubation at pH 6.6 increased low-affinity Cu²⁺ binding to A β 1-42, precipitation of A β 1-42 at the lower pH (Fig. 3D) was not increased compared with precipitation at pH 7.4 (Fig. 3B).

Although at pH 7.4 CDTA did not permit sufficient Cu²⁺ to bind to A β 1-42 to induce peptide precipitation (Fig. 3B), at pH 6.6 incubation of A β 1-42 with CDTA:Cu²⁺ complexes was accompanied by $\approx 50\%$ precipitation of A β 1-42 (Fig. 3D). Note, however, that CDTA and all of the chelator array have lower Cu²⁺ binding affinities at the lower pH; the $\log K_{app}$ of CDTA for Cu²⁺ decreases from 17.0 at pH 7.4 to 16.2 at pH 6.6 (Table 1).

Taken together, these findings indicate that A β 1-40 and A β 1-42 precipitate if bound to 1 Cu²⁺. Conversely, A β 1-40, and even A β 1-42 (at pH 7.4), will remain soluble if Cu²⁺ binding is totally prevented.

Although there was a strict correspondence between the stoichiometry of bound Cu²⁺ and the amount of A β 1-40 precipitation ($r^2 = 0.89$ at pH 7.4 and 0.85 at pH 6.6; Fig. 4), there was a weak correlation (and at pH 6.6, no correlation) between the stoichiometry of bound Cu²⁺ and the amount of A β 1-42 precipitation ($r^2 = 0.17$ at pH 7.4 and $r^2 = 0.09$ at pH 6.6; Fig. 4). The change in pH from 7.4 to 6.6 did not alter the slope of the linear relationship between Cu²⁺:A β 1-40 stoichiometry and precipitation (Fig. 4), which indicates that the Cu²⁺ binding to A β 1-40 saturates at 2.5 Cu²⁺ ions.

TABLE 4. Competition between Cu²⁺ and Zn²⁺ for metal ion binding sites on A β 1–40 at pH 7.4 and 6.6

	pH 7.4	pH 6.6
Zn ²⁺ + A β , 48 h		
% precipitation	88.4 \pm 0.9	85.0 \pm 0.4 ^a
Ratio Zn:A β	2.88 \pm 0.03	2.03 \pm 0.19 ^a
Cu ²⁺ + A β , 48 h		
% precipitation	58.0 \pm 3.9	85.4 \pm 6.0 ^a
Ratio Cu:A β	1.91 \pm 0.15	2.76 \pm 0.10 ^a
Zn ²⁺ + Cu ²⁺ coincubated for 48 h		
% precipitation	79.0 \pm 3.8	64.0 \pm 7.2 ^b
Ratio Zn:A β	1.90 \pm 0.18	0.33 \pm 0.09 ^a
Ratio Cu:A β	1.68 \pm 0.09	3.13 \pm 0.05 ^a
Total metal:A β ratio	3.58	3.46
Zn ²⁺ for 24 h, then Cu ²⁺ for 24 h		
% precipitation	65.8 \pm 7.2	82.0 \pm 4.1 ^a
Ratio Zn:A β	1.74 \pm 0.29	0.67 \pm 0.22 ^a
Ratio Cu:A β	1.48 \pm 0.09	2.75 \pm 0.04 ^a
Total metal:A β ratio	3.22	3.22

The following incubations were done: (a) A β 1–40 was incubated with 50 μ M Zn²⁺ for 48 h; (b) A β 1–40 was incubated with 50 μ M Cu²⁺ for 48 h; (c) A β 1–40 was incubated with 50 μ M Zn²⁺ and 50 μ M Cu²⁺ for 48 h; or (d) A β 1–40 was incubated with 50 μ M Zn²⁺ for 24 h, 50 μ M Cu²⁺ was added, and the mixture was incubated for a further 24 h. Stoichiometry represents metal ion binding to total (precipitated and soluble) A β 1–40. All experiments were done in Tris-buffered saline. Data are mean \pm SD values (n = 6).

The statistical significance of comparisons between pH 7.4 and 6.6 for percent precipitation and ratio of metal:A β for each experiment was determined by *t* test (two-tailed, heteroscedastic): ^a*p* < 0.001, ^b*p* < 0.005.

though the aggregated A β 1–42 bound 0.7 more Cu²⁺ at pH 6.6 than at pH 7.4.

Cu²⁺ initiates the nucleated precipitation of A β 1–40 and A β 1–42

Because, unlike A β 1–40, the precipitation of A β 1–42 did not correlate with the stoichiometry of bound Cu²⁺, we studied the possibility that the precipitation of A β 1–42 was by a seeding mechanism (Jarrett et al., 1993) where Cu²⁺ initiates peptide nucleation but that the precipitation of the majority of the solution was not metal-dependent. First, we measured the amount of precipitation of A β in the absence of added metal ions or chelators, incubated for 24 h under the same conditions as Fig. 3 and Table 3. The precipitation of A β 1–40 and A β 1–42 formed by an apparent seeding mechanism in the absence of metal ions or chelators was greater at pH 6.6 (23 \pm 7 and 95 \pm 4%, respectively) than at pH 7.4 (10 \pm 2 and 87 \pm 1%), in agreement with our previous report (Atwood et al., 1998). It is surprising that the precipitation of A β 1–40 or A β 1–42 at pH 6.6 in the absence of added metal ions was greater than the corresponding amount of A β 1–40 or A β 1–42 precipitation that occurred in the presence of 50 μ M Cu²⁺ that had been complexed with any of the high-affinity chelators (log *K*_{app} \geq 10.6; Fig. 3B and D). To illustrate this, in Fig. 5A we compare the precipitation of A β 1–40 and A β 1–42 in the presence and absence of the DTPA:Cu²⁺ complex. We studied DTPA because NMR spectroscopy

of DTPA coincubated with A β 1–40 and A β 1–42 indicated that the chelator does not bind to the peptide (X. Huang, K. Barnham, C. S. Atwood, R. E. Tanzi, and A. I. Bush, unpublished data); hence, any effect of DTPA in preventing A β precipitation is not due to β -sheet chain breaking. We observed that there is more precipitation of either A β 1–40 or A β 1–42 in the absence of added metal ions than in the presence of DTPA:Cu²⁺ complexes, at either pH (Fig. 5A). This indicates that the increased precipitation of A β at pH 6.6 in the absence of added metal ions could not be explained by pH effects alone and suggests that trace metal ion contamination of the buffers mediates the apparent seeding of A β solutions. Supporting this possibility, we measured the contaminating concentration of Cu²⁺ in buffer solutions containing A β as \sim 0.1 μ M, using inductive coupled plasma mass spectrometry.

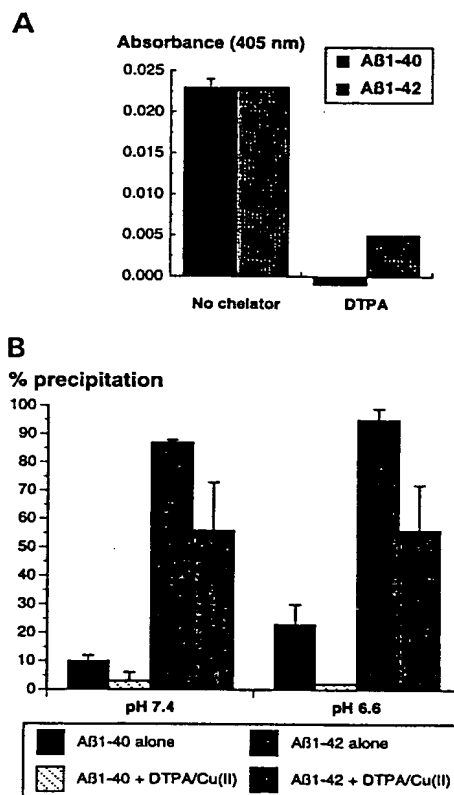


FIG. 5. Inhibition of A β nucleated precipitation by DTPA. **A:** A β 1–40 or A β 1–42 (10 μ M) was incubated at pH 7.4 and 6.6 with or without Cu²⁺:DTPA complex (chelator complex data selected from Fig. 3B and D) for 24 h, and precipitation was determined as in Fig. 3. Data are mean \pm SD (bars) values (n = 6). **B:** A β 1–40 or A β 1–42 (10 μ M each) was incubated in phosphate-buffered saline (pH 7.4) with or without DTPA (200 μ M) for 5 days at 37°C. Precipitation was assessed by measuring the turbidity of the solution at 405 nm. Data are mean \pm SD (bars) values (n = 4).

DISCUSSION

CMCA

The CMCA technique was developed for the practical assessment of stoichiometry and affinities of metal ion binding to soluble and precipitated proteins. Validation of our methodology was obtained by a comparison of Cu²⁺ and Zn²⁺ binding affinities with the known affinities for DSA (Fig. 2). This technique circumvents several problems associated with preexisting methodologies for the assessment of metal ion binding to proteins. Competition studies often require the use of expensive and hazardous radioactive metal ions or ligands, and spectrophotometric or fluorescence techniques lack sensitivity in the submicromolar metal ion concentration range and rely on conformational changes in the target protein.

Despite these advantages, the CMCA method relies on the availability of an array of chelators that reflect log K_{app} values within the range of interest. To this end, we attempted to provide chelators at log K_{app} intervals of no more than 0.5 units, but the number of usable chelators with log $K_{app} > 10$ was small. The array of data points required by this technique should be improved as we identify further appropriate chelators for use in the assay.

Binding interactions with Cu²⁺ differentiate A β 1-40 and A β 1-42

Our current results, summarized in Table 2, indicated multiple affinity Cu²⁺ binding sites on A β 1-40 and A β 1-42 that were considerably greater than the single affinities (4.0 and 0.3 μ M, respectively) initially obtained for the peptides using spectrophotometric analysis (Atwood et al., 1998). This apparent discrepancy is probably because, in the previous study, the precipitation of the peptide by Cu²⁺ withdrew a fraction of the peptide from spectral analysis and may have artifactually lowered the sensitivity of the technique to detect physicochemical changes. Also, the spectrometric analysis we used previously would have been only capable of appreciating spectral shifts associated with saturation of the lower-affinity Cu²⁺ binding site on A β . This is because in our previous analysis we measured the spectroscopic response of synthetic peptide to increments of ≥ 0.1 μ M in Cu²⁺ concentration. Because the background contamination of the incubation buffer with Cu²⁺ is ~ 0.1 μ M and because we have now identified Cu²⁺ binding sites on A β with subnanomolar affinities, the previous technique could not have reliably titrated the Cu²⁺ concentration in low nanomolar increments to appreciate a consequent spectroscopic effect. An advantage of the current CMCA technique was that we could titrate the physicochemical effects of the exchange of Cu²⁺ to A β against the known binding affinities of chelators in competition for Cu²⁺, without the need to titrate metal ion concentrations that are below background contamination levels.

The estimated log K_{app} for the Cu²⁺ binding site on A β 1-42 (log $K_{app} = 17.2$; Table 2) translates into a predicted affinity for Cu²⁺ in the attomolar range and for

A β 1-40 in the picomolar range (estimated log $K_{app} = 10.3$; Table 2), indicating that Cu²⁺ could be bound to A β (A β 1-42 \gg A β 1-40) under biological conditions. Also, the concave acceleration of the Scatchard analyses of Cu²⁺ binding to A β 1-40 and A β 1-42 (Fig. 3E) suggests negative cooperativity of the binding sites. A β 1-42 was found to have significantly less hindrance in binding successive Cu²⁺ ions compared with A β 1-40. A β 1-42 also exhibits higher affinity Cu²⁺ binding than A β 1-40 at both the lowest- and highest-affinity binding sites observed, which indicates that the structural basis for Cu²⁺ binding and the allosteric basis for cooperativity may be mediated by the hydrophobic carboxyl-terminal tail of A β . A β 1-42 reportedly has a higher β -sheet content than A β 1-40 (Barrow et al., 1992), and β -sheet or β -barrel conformations are known to mediate other high-affinity Cu binding sites (Frausto da Silva and Williams, 1993). The increased β -sheet content of A β 1-42 makes the peptide more liable to self-associate and to precipitate (Jarrett et al., 1993). This self-association could have an allosteric impact on Cu²⁺ binding, especially if Cu²⁺ binding to A β involves multimeric interactions between protein subunits. It is also possible that nucleated precipitation of A β 1-42 (see below) may have perturbed the equilibrium of bound to free soluble Cu²⁺ as the Cu²⁺ is removed from the soluble phase, thus contributing to the log K_{app} value we have observed. However, the possibility of irreversible withdrawal of Cu²⁺ associated with the precipitating peptide was not supported by our observation that high-affinity Cu chelators resolubilized Cu²⁺-associated A β 1-42 deposits (Fig. 6). It is also unlikely that our results only reflect Cu²⁺ binding to preassociated A β because the precipitation of soluble A β in the presence of Cu²⁺ occurs within seconds (Atwood et al., 1998) but over days in the absence of added metal ions (Jarrett et al., 1993). Nevertheless, preliminary data indicate Cu²⁺ binds to fibrillized A β with approximately the same stoichiometry as to fresh soluble A β (C. S. Atwood et al., unpublished data).

The structures of the multiple Cu²⁺ binding sites on soluble and precipitated A β are not yet clear and could be complex because new sites may form during the assembly of the peptide. Some evidence suggests that A β forms a dimer in solution (Garzon-Rodriguez et al., 1997; Huang et al., 1997). Therefore, the noninteger stoichiometry of 0.5 equivalents that was clearly observed for the highest-affinity binding site on A β 1-42 and 2.5 equivalents for the lowest-affinity Cu²⁺ binding site on both A β 1-40 and A β 1-42 at pH 6.6 (Fig. 3E and Table 2) may reflect interaction with A β dimers. It is also possible that some binding sites are created by residues on neighboring A β subunits that are only brought into Cu²⁺-coordinating alignment as the peptide aggregates.

We have also shown for the first time that Cu²⁺ slowly modifies A β , inducing SDS resistance (Fig. 7). Previous studies have shown that Fe/H₂O₂ oxidation systems promote SDS-resistant polymerization of A β generated in a wheat germ expression system (Dyrks et al., 1992), but

deprotonates and selectively coordinates to Cu²⁺. Our recent electron paramagnetic resonance evidence has indicated a coordination sphere for Cu²⁺ to A β of CuN₃O₁ (Huang et al., 1999b), and detailed simulations reveal that only two of these nitrogens are His residues, whereas the third is a deprotonated amide-N. When Cu²⁺ coordinates to an amide nitrogen, it is able to increase dramatically the acidity of the amide NH to <7 and as low as 4, whereas the pK_a of amides bound to Zn²⁺ is still ~12 (Sigel and Martin, 1982). This selective stabilization of a deprotonated (anionic) amide is the result of more effective ligand-to-metal charge transfer from the deprotonated amide nitrogen to the d⁹ Cu²⁺ versus a d¹⁰ Zn²⁺ transition metal ion. This is a common phenomenon among other electrophilic transition metal ions with vacant d orbitals (Woon and Fairlie, 1992). Therefore, we propose that although His residues may contribute to the coordination of both Cu²⁺ and Zn²⁺ sites, one amide group may coordinate an additional low-affinity Cu²⁺ as the pH is lowered from 7.4 to 6.6, whereas high-affinity Cu²⁺ coordination and Zn²⁺ coordination may be simultaneously weakened. A similar increase in Cu²⁺ binding in response to acidic pH (pH 3–6) has recently been reported for the multicopper oxidase apoFet3p (Davis-Kaplan et al., 1998).

Possible physiological and pathological roles for the selective Cu²⁺ and Zn²⁺ binding sites on A β

Our data indicate that A β binds approximately equivalent amounts of Cu²⁺ and Zn²⁺ at pH 7.4 but that Cu²⁺ totally displaces Zn²⁺ binding to A β at pH 6.6. We have previously published the finding that at pH 7.4 the Zn²⁺ binding site is very highly selective for Zn²⁺ and that at pH 7.4 Zn²⁺ cannot be displaced from binding A β by overwhelming concentrations of Cu²⁺ or any other transition metal ion (Bush et al., 1994a). Taken together with our current results, we hypothesize that A β possesses highly selective Cu²⁺ and Zn²⁺ binding sites at pH 7.4 but that at pH 6.6 there is a weakened affinity for Zn²⁺ but a preserved affinity for Cu²⁺ that allows the peptide to become occupied by Cu²⁺ at both sites. Thus, excessive Cu²⁺ binding of A β may be a consequence of mild acidosis and may have a deleterious promoting effect on the aggregation and the redox activities (Huang et al., 1999a,b; present study, Fig. 7) of the protein.

Low-affinity metal binding sites on A β mediate aggregation and precipitation and also may mediate Cu²⁺ reduction, O₂-dependent H₂O₂ production (Huang et al., 1999a), and oxidative modification of A β (Fig. 7). It is possible that one of the low-affinity Cu²⁺ sites we have observed may be due to Cu²⁺ occupying the Zn²⁺ site on A β . Abnormal Cu²⁺ binding to the Zn²⁺ binding site on wild-type and mutant superoxide dismutase has been shown to alter the redox chemistry of this protein and to promote radical generation, which may play a pathogenic role in familial amyotrophic lateral sclerosis (Goto et al., 1998). We contemplate similarities between this abnor-

mal neurochemistry in familial amyotrophic lateral sclerosis and abnormal A β -metal interactions in AD.

Abnormal Cu/Zn interactions with A β in AD

Zn and Cu ions mediate the precipitation of A β deposits in AD brain as evidenced by the solubilization of A β from postmortem AD brain tissue that is promoted by chelators selective for these metals (Cherny et al., 1999) and also evidenced by the marked enrichment of total Cu and Zn in AD neuropil and amyloid plaques (Lovell et al., 1998), which could be explained by the high affinity of these metal ions for A β (present study, Fig. 3 and Table 2; Bush et al., 1994a; Atwood et al., 1998). It is not yet clear whether abnormal Cu (or Zn) homeostasis initiates A β deposition, or whether the accumulation of A β acts as a sink that draws metal ions into its mass. Significant increases in the concentration of Cu in the CSF [2.2-fold (Basun et al., 1991)] accompanied by an increase in level of CSF and brain ceruloplasmin, a Cu transport protein, in AD patients (Loeffler et al., 1994) further indicate that Cu homeostasis is compromised in AD.

Both Cu and Zn are released from vesicles of neurons during synaptic transmission reaching concentrations as high as 15 μ M (Hartter and Barnea, 1988) and 300 μ M (Frederickson, 1989), respectively. The proportion of binding of Cu and Zn to A β will depend on the respective stability constants of each metal ion for different ligands, their total concentrations, and the pH of the microenvironment. The release of Cu and Fe from metalloproteins is usually promoted by mildly acidic environments (reviewed by Atwood et al., 1998). However, under mildly acidic conditions Cu²⁺ binding to A β was enhanced, almost completely displacing Zn²⁺ from A β (Table 3). This unusual property of A β to accept Cu²⁺ under mildly acidic conditions, where most proteins lose metal ions with decreasing pH, may be important in the pathophysiology of AD, where metabolic compromise may result in acidosis (Yates et al., 1990).

Acknowledgment: The authors thank Diane Cabelli, Alan Kay, J. D. Robertson, and Kevin Barnham for helpful discussions. This work is supported by funds from the National Institutes of Health (grant R29AG12686), PRANA Corp., and the American Health Assistance Foundation.

REFERENCES

- Appleton D. W. and Sarkar B. (1971) The absence of specific copper (II)-binding site in dog albumin. *J. Biol. Chem.* 246, 5040–5046.
- Atwood C. S., Moir R. D., Huang X., Bacarra N. M. E., Scarpa R. C., Romano D. M., Hartshorn M. A., Tanzi R. E., and Bush A. I. (1998) Dramatic aggregation of Alzheimer Abeta by Cu(II) is induced by conditions representing physiological acidosis. *J. Biol. Chem.* 273, 12817–12826.
- Barrow C. J., Yasuda A., Kenny P. T., and Zagorski M. G. (1992) Solution conformations and aggregational properties of synthetic amyloid beta-peptides of Alzheimer's disease. Analysis of circular dichroism spectra. *J. Mol. Biol.* 225, 1075–1093.
- Basun H., Forsell L. G., Wetterberg L., and Winblad B. (1991) Metals and trace elements in plasma and cerebrospinal fluid in normal

- Soto C., Brañes M. C., Alvarez J., and Inestrosa N. C. (1994) Structural determinants of the Alzheimer's amyloid β -peptide. *J. Neurochem.* **63**, 1191–1198.
- Spiro T. G. and Saltman P. (1969) *Structure and Bonding*, Vol. 6. Springer-Verlag, New York.
- Suh S. W., Jensen K. B., Jensen M. S., Silva D. S., Kesslak J. P., Danscher G., and Frederickson C. J. (2000) Histological evidence implicating zinc in Alzheimer's disease. *Brain Res.* **852**, 274–278.
- Thompson C. M., Markesbery W. R., Alaudin M., Hossain T. I. M., and Brubaker E. H. (1988) Regional brain trace-element studies in Alzheimer's disease. *Neurotoxicology* **9**, 1–8.
- Vance D. E., Ehmann W. D., and Markesbery W. R. (1990) A search for longitudinal variations in trace element levels in nails of Alzheimer's disease patients. *Biol. Trace Element Res.* **26–27**, 461–470.
- Vigo-Pelfrey C., Lee D., Keim P., Lieberburg I., and Schenk D. B. (1993) Characterization of β -amyloid peptide from human cerebrospinal fluid. *J. Neurochem.* **61**, 1965–1968.
- Woon T. C. and Fairlie D. P. (1992) Amide complexes of diethylenetriamineplatinum(II). *Inorg. Chem.* **31**, 4069–4074.
- Yates C. M., Butterworth J., Tennant M. C., and Gordon A. (1990) Enzyme activities in relation to pH and lactate in postmortem brain in Alzheimer-type and other dementia. *J. Neurochem.* **55**, 1624–1630.